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(54) Title: PHOSPHOLIPASE FROM ZYGOASCUS HELLENICUS

(57) Abstract: The inventors discovered and isolated a phospholipase from Zygoascus *hellenicus*. The inventors also isolated the gene encoding the novel phospholipase and cloned it into an *E. coli* strain.

PHOSPHOLIPASE FROM ZYGOASCUS HELLENICUS

FIELD OF THE INVENTION

The present invention relates to a phospholipase, methods of using and producing it, as well as a nucleic acid sequence encoding it.

BACKGROUND OF THE INVENTION

Phospholipases are known to be useful, e.g. in baking and oil degumming.

Fu et al., Microbiology 143, 331-340 (1997) describe the cloning and characterization of a gene (LIP1) which encodes a lipase from the pathogenic yeast *Candida albicans* and state that the lipase has no phospholipase activity. The GenBank accession number for LIP1 is U34807.

LIP4 (EMBL, GenBank AF 191317) is described as a secreted lipase from *Candida albicans*.

Molecular cloning and expression of phospholipase B genes from *Candida albicans* has been described. Sugiyama et al., Medical Mycology, 37 (1), 61-67 (1999). Hoover et al., FEMS Microbiology Letters, 167 (2), 163-169 (1998). Leidich et al., J. Biological Chemistry, 273 (40), 26078-26086 (1998). GenBank AL033501.

SUMMARY OF THE INVENTION

The inventors discovered and isolated a phospholipase from *Zygoascus hellenicus*. The inventors also isolated and sequenced the gene encoding the novel phospholipase and cloned it into an *E. coli* strain.

Accordingly, the invention provides a phospholipase which may be a polypeptide having an amino acid sequence as the mature peptide shown in SEQ ID NO: 1 or a polypeptide encoded by the phospholipase encoding part of the DNA sequence cloned into a plasmid present in *E. coli* DSM 13648.

The phospholipase may also be an analogue of the polypeptide defined above which:

i) has at least 50 % identity with said polypeptide,

ii) is immunologically reactive with an antibody raised against said polypeptide in purified form,

iii) is an allelic variant of said polypeptide,

Finally, the phospholipase of the invention may be a polypeptide which is encoded by a nucleic acid sequence which hybridizes at 55°C with a complementary strand of the nucleic acid sequence of SEQ ID NO: 1 encoding the mature polypeptide or a subsequence thereof having at least 100 nucleotides.

The nucleic acid sequence of the invention may comprise a nucleic acid sequence which encodes the phospholipase described above, or it may encode a phospholipase and comprise:

a) the DNA sequence encoding a mature phospholipase cloned into a plasmid present in *Escherichia coli* DSM 13648,

b) the DNA sequence encoding a mature phospholipase shown in SEQ ID NO: 1, or

c) an analogue of the DNA sequence defined in a) or b) which

i) has at least 60 % identity with said DNA sequence, or

ii) hybridizes at 55°C with said DNA sequence, its complementary strand or a subsequence thereof.

Other aspects of the invention provide a recombinant expression vector comprising the DNA sequence, and a cell transformed with the DNA sequence or the recombinant expression vector.

A comparison with known sequences shows that the closest prior-art sequence is LIP4 (EMBL, GenBank AF 191317), described as a secreted lipase from *Candida albicans*. The identity was calculated to be 38 % for the mature proteins and 52 % for DNA sequences encoding the mature proteins, disregarding the STOP codons.

DETAILED DESCRIPTION OF THE INVENTION

Genomic DNA source

A phospholipase of the invention may be derived from a strain of *Zygoascus*, particularly *Z. hellenicus*, e.g. strain CBS 4075, using probes designed on the basis of the DNA sequences in this specification. The strain is commercially available from Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.

A strain of *Escherichia coli* containing a gene encoding phospholipase was deposited by the inventors under the terms of the Budapest Treaty with the DSMZ - Deutshe Sammmlung von Microorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig DE, Germany. The deposit date was 10 August 2000, and the accession number was DSM 13648. The deposit was made by Novo Nordisk A/S and was later assigned to Novozymes A/S.

Properties of phospholipase

The phospholipase has phospholipase A activity and is able to hydrolyze lecithin by releasing fatty acid. It has no lipase activity and shows no activity towards tributyrin. The isoelectric point is around 4.2.

Recombinant expression vector

The expression vector of the invention typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a selectable marker, a transcription terminator, a repressor gene or various activator genes. The vector may be an autonomously replicating vector, or it may be integrated into the host cell genome.

Production by cultivation of transformant

The phospholipase of the invention may be produced by transforming a suitable host cell with a DNA sequence encoding the phospholipase, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

The host organism is particularly a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, such as a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, particularly *A. niger*, *A. oryzae*, *F. graminearum*, *F. sambucinum*, *F. cerealis* or *S. cerevisiae*, e.g. a glucoamylase-producing strain of *A. niger* such as those described in US 3677902 or a mutant thereof. The production of the lysophospholipase in such host organisms may be done by the general methods described in EP 238,023 (Novo Nordisk), WO 96/00787 (Novo Nordisk) or EP 244,234 (Alko).

Hybridization

The hybridization is used to indicate that a given DNA sequence is analogous to a nucleotide probe corresponding to a DNA sequence of the invention. The hybridization conditions are described in detail below.

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at approx. 45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5 % SDS at a temperature of at least 55°C, more particularly at least 60°C, more particularly at least 65°C, even more particularly at least 70°C, especially at least 75°C.

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using a x-ray film.

Alignment and identity

The phospholipase and the nucleotide sequence of the invention may have identities to the disclosed sequences of at least 60 % or at least 70 %, e.g. at least 85 %, particularly at least 90 % or at least 95 %, e.g. at least 98 %.

For purposes of the present invention, alignments of sequences and calculation of identity scores were done using the software Align, a Needleman-Wunsch alignment (i.e. global alignment), useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in a gap is -2 for proteins and -4 for DNA. Align is from the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98).

Use of phospholipase

The phospholipase of the invention can be used in various industrial application of phospholipases, e.g. as described below.

Use in baking

The phospholipase of the invention can be used in the preparation of dough and baked products (such as bread and cakes), e.g. to achieve a larger loaf volume of the baked product and/or a better shape retention during baking and/or to improve the elasticity of the baked product. Thus, the phospholipase can be used in a process for making bread, comprising adding the phospholipase to the ingredients of a dough, kneading the dough and baking the dough to make the bread. This can be done in analogy with US 4567056 or WO 99/53769.

Use in detergent

The variant may be used as a detergent additive, e.g. at a concentration (expressed as pure enzyme protein) of 0.001-10 (e.g. 0.01-1) mg per gram of detergent or 0.001-100 (e.g. 0.01-10) mg per liter of wash liquor.

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations. In a laundry detergent, the variant may be effective for the removal of fatty stains, for whiteness maintenance and for dingy cleanup. A laundry detergent composition may be formulated as described in GB 2247025, WO 9901531 or WO 9903962.

The detergent composition of the invention may particularly be formulated for hand or machine dishwashing operations. e.g. as described in GB 2,247,025 (Unilever) or WO 99/01531 (Procter & Gamble). In a dishwashing composition, the variant may be effective for removal of greasy/oily stains, for prevention of the staining /discoloration of the dishware and plastic components of the dishwasher by highly colored components and the avoidance of lime soap deposits on the dishware.

Other uses

The phospholipase of the invention can be used to improve the filterability of an aqueous solution or slurry of carbohydrate origin by treating it with the phospholipase. This is particularly applicable to a solution of slurry containing a starch hydrolyzate, especially a wheat starch hydrolyzate, since this tends to be difficult to filter and to give cloudy filtrates. The treatment can be done in analogy with EP 219,269 (CPC International).

Further, the phospholipase of the invention may be used for partial hydrolysis of phospholipids, particularly lecithin, to obtain improved phospholipid emulsifiers. This application is further described in Ullmann's Encyclopedia of Industrial Chemistry (Publisher: VCH Weinheim (1996)), JP patent 2794574, and JP-B 6-087751.

Further, the phospholipase of the invention may be used in a process for the production of an animal feed which comprises mixing the phospholipase with feed substances and at least one phospholipid. This can be done in analogy with EP 743 017.

Even further the phospholipase of the invention can be used in a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the phospholipase so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil. This process is applicable to the purification of any edible oil which contains phospholipid, e.g. vegetable oil such as soy bean oil, rape seed oil and sunflower oil. JP-A 2-153997, US 5264367.

The phospholipase can also be used for making cheese as described in WO 00/54601.

EXAMPLES

Materials and methods

Methods

Unless otherwise stated, DNA manipulations and transformations were performed using standard methods of molecular biology as described in Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology", John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.).

Enzymes

Enzymes for DNA manipulations (*e.g.* restriction endonucleases, ligases etc.) are obtainable from New England Biolabs, Inc. and were used according to the manufacturer's instructions.

Plasmids/vectors

pT7Blue (Invitrogen, Netherlands)

Cloning

LA PCR™ in vitro Cloning Kit (TaKaRa) was used for cloning according to the manufacturer's instructions.

Microbial strains

E. coli JM109 (TOYOBO, Japan)

E.coli DB6507 (F,pnrF74::Tn5,seupE44, lacY1, ara-14,galK2, xyl-5, mtl-1, leuB6, proA2, hsdS20, recA13, rpsL20, thi-1, lambda-)

Media and reagents

Cove: 342.3 g/L Sucrose, 20 ml/L COVE salt solution, 10mM Acetamide, 30 g/L noble agar.

Cove-2: 30 g/L Sucrose, 20 ml/L COVE salt solution, 10mM, Acetamide, 30 g/L noble agar.

Cove salt solution: per liter 26 g KCl, 26 g MgSO4-7aq, 76 g KH2PO4, 50ml Cove trace metals.

Cove trace metals: per liter 0.04 g NaB4O7-10aq, 0.4 g CuSO4-5aq, 1.2 g FeSO4-7aq, 0.7 g MnSO4-aq, 0.7 g Na2MoO2-2aq, 0.7 g ZnSO4-7aq.

AMG trace metals: per liter 14.3 g ZnSO4-7aq, 2.5 g CuSO4-5aq, 0.5 g NiCl2, 13.8 g FeSO4, 8.5 g MnSO4, 3.0 g citric acid.

YPG: 4 g/L Yeast extract, 1 g/L KH2PO4, 0.5 g/L MgSO4-7aq, 5 g/L Glucose, pH 6.0.

STC: 0.8 M Sorbitol, 25 mM Tris pH 8, 25 mM CaCl2.

STPC: 40 % PEG4000 in STC buffer.

Cove top agarose: 342.3 g/L Sucrose, 20 ml/L COVE salt solution, 10mM Acetamide, 10 g/L low melt agarose.

MLC : per liter 40 g glucose, 50 g soybean powder, 4 g citric acid, pH 5.0.

MU-1: per liter 260 g maltodextrin, 3 g MgSO4-7aq, 6 g K2SO4, 5 g KH2PO4, 0.5 ml Trace metal solution for AMG (MU-1)

Trace metal solution for AMG (MU-1) : per 100 ml 1.39 g FeSO4-7aq, 1.356 g MnSO4-5aq, 0.68 g ZnCl2, 0.25 g CuSO4-5aq, 0.024 g NiCl2-6aq, 0.3 g Citric acid

MT-O medium

Soybean powder	30 g/liter
Corn steep powder	5 g/liter
Yeast extract	1 g/liter
Peptone	5 g/liter
Glucose	10 g/liter
NH4NO3	2.5 g/liter
K2HPO4	4 g/liter
MgSO4 7 H2O	0.1 g/liter
Olive oil	2 ml/shake flask
Adeka Pluronic L-61	2 drops/shake flask

Deionized water is used. pH is adjusted to 6.5 before autoclaving. 100 ml substrate/shake flask (500 ml). Adeka Pluronic L-61 is an antifoam from Asahi Denka Kogyo K.K. (Japan).

Lecithin plates

Substrate: 0.5 % L-alpha Phosphatidyl Choline 95% (AVANTI 441601).
Emulsifier: 0.25% Cholic acid
Buffer: 50mM acetate buffer pH5.5
10mM CaCl2
Agar Noble 2%
2% Brilliant Green 15ml/L

Example 1: Production of phospholipase

Zygoascus hellenicus CBS 4075 was grown for 4 days at 25°C on PDA slants (product of Difco with 1 % extra agar added).

Cells were harvested in 0.1% Tween 20 water and 57 MT-O shake flasks were inoculated and incubated on a rotary shaker at 30°C for 3 days.

Cells and particulate matter were removed by centrifugation giving 4.4 liter of supernatant. The supernatant was concentrated by ultrafiltration, washed with ca. 2 volumes of deionized water and freezedried.

About 40 g of freeze-dried powder was obtained.

Example 2: Purification of wild type Phospholipase

1-gram powder from Example 1 was dissolved in 100 ml water and ultra filtrated using Amicon filter (YM10 kDa cut off). After concentration to 10 ml, pH of the solution was adjusted to 8 and conductivity was 2 mSi.

The sample was then applied on anion exchanger FFQ sepharose equilibrated with 50 mM Borate buffer pH 8. Bound enzyme was eluted using linear salt gradient using same buffer containing 1 M salt. Fractions containing protein were collected.

SDS-PAGE of the fractions showed a single band.

N-terminal of the blotted protein was determined. The sequence of the N-terminal was as shown in SEQ ID NO: 8.

The enzyme was qualitatively assayed to confirm phospholipase activity using Lecithin as substrate and NEFA-kit from Waco chemicals to determine free fatty acids released.

Example 3: Cloning and expression of lipase gene from *Zygoascus hellenicus*

Cloning of phospholipase gene from *Zygoascus hellenicus*

A strain of *Zygoascus hellenicus* was used as a genomic DNA supplier. Cloning was carried out with adaptor PCR method using LA PCR™ in vitro Cloning Kit (TaKaRa)

PCR reactions on *Zygoasucus hellenicus* genomic DNA which was digested by Pst I and ligated to Pst I cassette of the kit was done with ZhelN-2 (SEQ ID NO: 3) designed from N- terminal amino acid sequence of purified protein.

Reaction components (20ng /µl of genomic DNA with cassette, 1 mM dNTP each, primer 2 nM each, 2.5mM MgCl2, 0.5 U/ µl in LA Taq polymerase in 1X buffer (TaKaRa, Japan)) were mixed and submitted to PCR under the following conditions.

Step	Temperature	Time
1	94°C	30sec
2	50°C	2 min
3	72°C	1 min
4	4°C	forever

Steps 1 to 3 were repeated 30 times.

Amplified 640bp of the fragment was purified by GFX™ PCR DNA and Gel Band Purification kit (Amersham Pharmacia Biotech) and sequenced.

In order to clone the missing part of the gene, genome walking was done.

N-terminal part was obtained from the reaction mixture; 20 ng/µl of genomic DNA with BamH I cassette, 1 mM dNTP each, 2 nM, ZNr-2 primer, 2 nM, cassette primer, 2.5mM MgCl2, 0.5 U/ µl in LA Taq polymerase in 1X buffer (TaKaRa, Japan).

ZNr-2: 5'-cttgtagagcagctggtaagatttc-3'

Step	Temperature	Time
1	94°C	30sec
2	55°C	2 min
3	72°C	1 min
4	4°C	forever

Steps 1 to 3 were repeated 30 times.

C-terminal part was obtained by nested PCR with the primers ZC-1 and ZC-2 (SEQ ID NOS: 4 and 5) and genomic DNA with BamH I cassette. Reaction condition was same as above.

Obtained fragments were purified by GFX™ PCR DNA and Gel Band Purification kit (amersham pharmacia biotech) and sequenced with each primer which amplified the fragment.

The fidelity of LA-taq polymerase is not so good so in order to get the right sequence whole gene was amplified the primers Zhel-N (Bam) and Zhel-C (Sal) (SEQ ID NOS: 6 and 7).

Reaction components (6 ng /μl of genomic DNA, 250 mM dNTP each, primer 250 nM each, 0.05 U/ μl of Expand high fidelity polymerase in 1X buffer (Roche Diagnostics, Japan)) were mixed and submitted for PCR under the following conditions.

Step	Temperature	Time
1	94°C	2 min
2	94°C	10sec
3	55°C	30sec
4	68°C	45sec
step 2-4 repeat 10 times		
5	94°C	10sec
6	55°C	30sec
7	68°C	45sec +20sec/cycle
step 5-7, repeat 20 times		
8	68°C	7min
7	4°C	forever

An amplified DNA fragment was gel-purified with GFX™ PCR DNA and Gel Band Purification kit (Amersham Pharmacia Biotech) and ligated into a pT7Blue vector with ligation high (TOYOBO, Japan). The ligation mixtures were transformed into *E. coli* JM109. The resultant plasmids, pTZhel-2, pTZhel-5, pTZhel-6, and pTZhel-7, were sequenced and all of them are identical. The sequence is defined as *Zygoascus hellenicus* phospholipase DNA sequence.

Expression of phospholipase gene in *Aspergillus niger.*

The phospholipase gene was digested from pTZhel-5 with BamH I and Xho I and ligated into the BamH I and XhoI sites in the *Aspergillus* expression cassette pMT2188 which has *Aspergillus niger* neutral amylase promoter, *Aspergillus nidulans* TPI leader sequences,

Aspergillus niger glucoamylase terminator and *Aspergillus nidulans* amdS gene as a marker and Saccharomyces cerevisiae URA3 gene as a marker for a plasmid construction. The ligation mixture was transformed E.coli 6507 by electroporation and the resultant plasmid was pZPL-8.

A strain of *Aspergillus niger* was inoculated to 100 ml of YPG medium and incubated for16 hrs at 32°C at 120 rpm. Pellets were collected and washed with 0.6 M KCl, and resuspended 20 ml 0.6 M KCl containing a commercial β-glucanase product (Glucanex, product of Novo Nordisk A/S) at the concentration of 30 µl/ml. Cultures were incubated at 32°C at 60 rpm until protoplasts formed, then washed with STC buffer twice. The protoplasts were counted with a hematometer and resuspended in an 8:2:0.1 solution of STC:STPC:DMSO to a final concentration of 2.5x10e7 protoplasts/ml. About 3 µg of DNA was added to 100 µl of protoplasts solution, mixed gently and incubated on ice for 30 min. One ml of SPTC was added and incubated 30 min at 37°C. After the addition of 10 ml of 50°C Cove top agarose, the reaction was poured onto Cove agar plate. Transformation plates were incubated at 32°C for 5 days.

pZPL-8 was transformed into the strain of *Aspergillus niger*. The selected transformants were inoculated in 100 ml of MLC media and cultivated at 32°C for 2 day. 5 ml of grown cell in MLC medium was inoculated to 100 ml of MU-1 medium and cultivated at 32°C for 3 days. The supernatant was obtained by centrifugation.

The phospholipase activity was checked by a lecitin plate whose composition is described above.

The substrate and the emulsifier were dissolved together in appropriate amount of D.W and autoclaved briefly (120°C for 5min). The solution was stirred until the small particles had disappeared. Afterward it was subjected to hiscotron for 5min. It was mixed well with other components and 25ml aliquots were poured into Falcon 1004 plates.

Supernatant of transformants showed phospholipase activity with green halo on the assay plate.

Original (for SUBMISSION) - printed on 18.09.2001 09:06:24 AM

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.03.2001)
0-2	International Application No.	PCT/DK01/00600
0-3	Applicant's or agent's file reference	10078-WO

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	2
1-2	line	25-28
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	10 August 2000 (10.08.2000)
1-3-4	Accession Number	DSMZ 13648
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	Yes
0-4-1	Authorized officer	Marie Louise Rosendal Head Clerk

FOR INTERNATIONAL BUREAU USE ONLY

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CLAIMS

1. A phospholipase which is:

a) a polypeptide encoded by the phospholipase encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* deposit number DSM 13648, or

b) a polypeptide having an amino acid sequence as the mature peptide shown in SEQ ID NO: 1, or which can be obtained therefrom by substitution, deletion, and/or insertion of one or more amino acids;

c) an analogue of the polypeptide defined in (a) or (b) which:

i) has at least 50 % identity with said polypeptide,

ii) is immunologically reactive with an antibody raised against said polypeptide in purified form,

iii) is an allelic variant of said polypeptide,

d) a polypeptide which is encoded by a nucleic acid sequence which hybridizes at 55°C with a complementary strand of the nucleic acid sequence of SEQ ID NO: 1 encoding the mature polypeptide or a subsequence thereof having at least 100 nucleotides.

2. The phospholipase of claim 1 which is native to a strain of *Zygoascus*, particularly *Z. hellenicus*, more particularly strain CBS 4075.

3. A nucleic acid sequence comprising a nucleic acid sequence which encodes the phospholipase of claim 1 or 2.

4. A nucleic acid sequence which comprises:

a) the partial DNA sequence encoding a mature phospholipase cloned into a plasmid present in *Escherichia coli* DSM 13648,

b) the partial DNA sequence encoding a mature phospholipase shown in SEQ ID NO: 1,

c) an analogue of the sequence defined in a) or b) which encodes a phospholipase and

i) has at least 60 % identity with said DNA sequence, or

ii) hybridizes at 55°C with a complementary strand of said DNA sequence or a subsequence thereof having at least 100 nucleotides,

iii) is an allelic variant thereof, or

d) a complementary strand of a), b) or c).

5. A nucleic acid construct comprising the nucleic acid sequence of claim 3 or 4 operably linked to one or more control sequences capable of directing the expression of the phospholipase in a suitable expression host.

6. A recombinant expression vector comprising the nucleic acid construct of claim 5, a promoter, and transcriptional and translational stop signals.

7. A recombinant host cell comprising the nucleic acid construct of claim 6.

8. A method for producing a phospholipase comprising cultivating the host cell of claim 7 under conditions conducive to production of the phospholipase, and recovering the phospholipase.

9. A method for preparing a dough or a baked product made from the dough, comprising adding the phospholipase of claim 1 to the dough.

10. A dough composition comprising the phospholipase of claim 1.

11. A detergent composition comprising a surfactant and the phospholipase of claim 1.

12. A process for reducing the content of phosphorus in a vegetable oil, comprising contacting the oil with the phospholipase of claim 1 in the presence of water, and then separating an aqueous phase from the oil.

10078-WO.ST25
SEQUENCE LISTING

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gct aat ctt gat tca ctc gct gat ggt act att atc aga tct cga gaa      144
Ala Asn Leu Asp Ser Leu Ala Asp Gly Thr Ile Ile Arg Ser Arg Glu
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aca ccc aac tct ctt gct gca ttg gtg ctg ttc cct caa aac gtc cag      192
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Thr Pro Asn Ser Leu Ala Ala Leu Val Leu Phe Pro Gln Asn Val Gln
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aaa tct tac cag ctg ctc tac aag tct act gat gat ctg aat aga act      240
Lys Ser Tyr Gln Leu Leu Tyr Lys Ser Thr Asp Asp Leu Asn Arg Thr
        50                  55                  60

ttc gct aca gca acc acg atc att gtt ccc tat aac gct gat tat acc      288
Phe Ala Thr Ala Thr Thr Ile Ile Val Pro Tyr Asn Ala Asp Tyr Thr
    65                  70                  75

aag ctt gtc tcc ttc cag att gca gag gat tcg gcc tat atc aac tgt      336
Lys Leu Val Ser Phe Gln Ile Ala Glu Asp Ser Ala Tyr Ile Asn Cys
80                  85                  90                  95

gca ccc tct tat gcc ttg caa ttt gcc tca gac cct gat gct ctt ctt      384
Ala Pro Ser Tyr Ala Leu Gln Phe Ala Ser Asp Pro Asp Ala Leu Leu
                100                 105                 110

tca cag gtt gaa ttt gtt ttc cta tct gct atg ctt tca ctg gga tat      432
Ser Gln Val Glu Phe Val Phe Leu Ser Ala Met Leu Ser Leu Gly Tyr
            115                 120                 125

gtt gtg aat gtt cct gat tac gaa ggt ccg cga tct gct ttc act gta      480
Val Val Asn Val Pro Asp Tyr Glu Gly Pro Arg Ser Ala Phe Thr Val
        130                 135                 140

gga cag gtt gcc ggc aga gca gtc ttg gac tct ctt aga gca gct ctt      528
Gly Gln Val Ala Gly Arg Ala Val Leu Asp Ser Leu Arg Ala Ala Leu
    145                 150                 155

gca tcc tcg tct att aca gga atc aat tct aaa gct cgt att gcc ttg      576
Ala Ser Ser Ser Ile Thr Gly Ile Asn Ser Lys Ala Arg Ile Ala Leu
160                 165                 170                 175

tgg ggc tac tct ggt ggc tcc ctt gct agt ggc tgg gca gct cag ctt      624
Trp Gly Tyr Ser Gly Gly Ser Leu Ala Ser Gly Trp Ala Ala Gln Leu
                180                 185                 190

caa ccc tcg cat gcc cct gag ttg gtt atc tct ggt gct gct ttg ggt      672
Gln Pro Ser His Ala Pro Glu Leu Val Ile Ser Gly Ala Ala Leu Gly
            195                 200                 205

ggt gtt gta cct aat att gct gct gtc gca acc aag gtc agc ggt ggt      720
Gly Val Val Pro Asn Ile Ala Ala Val Ala Thr Lys Val Ser Gly Gly
        210                 215                 220

gta ttc gct ggc ttg gtt cct gca ggt atc acc ggt ttt ggt aac cag      768
Val Phe Ala Gly Leu Val Pro Ala Gly Ile Thr Gly Phe Gly Asn Gln
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tat gat gaa gtt agt gaa atc att tct gct cac ttg gtt cct tcc aag      816
Tyr Asp Glu Val Ser Glu Ile Ile Ser Ala His Leu Val Pro Ser Lys
240                 245                 250                 255

gtt gaa gag tgg aac aaa gct aat agt cag tgt atc gtt gct gat gct      864
Val Glu Glu Trp Asn Lys Ala Asn Ser Gln Cys Ile Val Ala Asp Ala
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tta gga tat gca ttc cag gat att ttc gag gat tat ttc act att ggt      912
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Thr Gly Ile Leu Glu Val Pro Glu Val Ala Ala Arg Leu Asn Gln Asn
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ctt atg ggt tct ttg gtt cct gaa ttt cct att tac att tac cat gct     1008
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10078-WO.ST25

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Leu Met Gly Ser Leu Val Pro Glu Phe Pro Ile Tyr Ile Tyr His Ala
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gtt ggc gac gaa gtt gct cct gtc gaa aaa gct gat act ctt gtc tct     1056
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320                 325                 330                 335

tcc tgg tgc aat gct ggt atc aaa tct ctt gaa tat cat aag gat att     1104
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ttg gat gag cat ctt tca gag gag gtg ttt ggt tca gct ggc gcc att     1152
Leu Asp Glu His Leu Ser Glu Glu Val Phe Gly Ser Ala Gly Ala Ile
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gtc tgg ttg aaa tcc atc atg gaa gac tct gct gat ctc gct gat ggc     1200
Val Trp Leu Lys Ser Ile Met Glu Asp Ser Ala Asp Leu Ala Asp Gly
        370                 375                 380

tgc aaa act gaa atc tct ctt tcg caa ctt att gat cct gat gct gtt     1248
Cys Lys Thr Glu Ile Ser Leu Ser Gln Leu Ile Asp Pro Asp Ala Val
    385                 390                 395

cct gtt ctt ggt gaa gag ttg gtg caa gtg ttg ctt aac tta gtt gga     1296
Pro Val Leu Gly Glu Glu Leu Val Gln Val Leu Leu Asn Leu Val Gly
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aag cct atc ggg ccc atc tcg ttt gcc taa                            1326
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Ala Gly Ile Pro Val Ala Ile Ser Asp Asp Pro Trp Tyr Asn Ala Pro
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Ala Asn Leu Asp Ser Leu Ala Asp Gly Thr Ile Ile Arg Ser Arg Glu
                20                  25                  30

Thr Pro Asn Ser Leu Ala Ala Leu Val Leu Phe Pro Gln Asn Val Gln
            35                  40                  45

Lys Ser Tyr Gln Leu Leu Tyr Lys Ser Thr Asp Asp Leu Asn Arg Thr
        50                  55                  60

Phe Ala Thr Ala Thr Thr Ile Ile Val Pro Tyr Asn Ala Asp Tyr Thr
    65                  70                  75
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Lys Leu Val Ser Phe Gln Ile Ala Glu Asp Ser Ala Tyr Ile Asn Cys
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Ala Pro Ser Tyr Ala Leu Gln Phe Ala Ser Asp Pro Asp Ala Leu Leu
                100                 105                 110

Ser Gln Val Glu Phe Val Phe Leu Ser Ala Met Leu Ser Leu Gly Tyr
            115                 120                 125

Val Val Asn Val Pro Asp Tyr Glu Gly Pro Arg Ser Ala Phe Thr Val
        130                 135                 140

Gly Gln Val Ala Gly Arg Ala Val Leu Asp Ser Leu Arg Ala Ala Leu
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Ala Ser Ser Ser Ile Thr Gly Ile Asn Ser Lys Ala Arg Ile Ala Leu
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Trp Gly Tyr Ser Gly Gly Ser Leu Ala Ser Gly Trp Ala Ala Gln Leu
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Gln Pro Ser His Ala Pro Glu Leu Val Ile Ser Gly Ala Ala Leu Gly
            195                 200                 205

Gly Val Val Pro Asn Ile Ala Ala Val Ala Thr Lys Val Ser Gly Gly
        210                 215                 220

Val Phe Ala Gly Leu Val Pro Ala Gly Ile Thr Gly Phe Gly Asn Gln
    225                 230                 235

Tyr Asp Glu Val Ser Glu Ile Ile Ser Ala His Leu Val Pro Ser Lys
240                 245                 250                 255

Val Glu Glu Trp Asn Lys Ala Asn Ser Gln Cys Ile Val Ala Asp Ala
                260                 265                 270

Leu Gly Tyr Ala Phe Gln Asp Ile Phe Glu Asp Tyr Phe Thr Ile Gly
            275                 280                 285

Thr Gly Ile Leu Glu Val Pro Glu Val Ala Ala Arg Leu Asn Gln Asn
        290                 295                 300

Leu Met Gly Ser Leu Val Pro Glu Phe Pro Ile Tyr Ile Tyr His Ala
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Val Gly Asp Glu Val Ala Pro Val Glu Lys Ala Asp Thr Leu Val Ser
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Ser Trp Cys Asn Ala Gly Ile Lys Ser Leu Glu Tyr His Lys Asp Ile
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Leu Asp Glu His Leu Ser Glu Glu Val Phe Gly Ser Ala Gly Ala Ile
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Val Trp Leu Lys Ser Ile Met Glu Asp Ser Ala Asp Leu Ala Asp Gly
370 375 380

Cys Lys Thr Glu Ile Ser Leu Ser Gln Leu Ile Asp Pro Asp Ala Val
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Pro Val Leu Gly Glu Glu Leu Val Gln Val Leu Leu Asn Leu Val Gly
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1 5 10 15

Asn Leu Asp Ser Leu Ala
20

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 01/00600

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/20 //C11D3/386,A21D8/04,A21D10/00,C11B3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1991:118793, vol. 28, no. 5, pages 355-362, MAGO N ET AL : "Subcellular localization of enzymes of phospholipid metabolism in candida-albicans" XP002902240 abstract & JOURNAL OF MEDICAL AND VETERINARY MYCLOLOGY, 1990, ---	1-12
A	WO 99 03962 A (BAECK ANDRE CESAR) 28 January 1999 (1999-01-28) page 3 -page 5 --- -/--	11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 January 2002	22 02 2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Yvonne Siösteen

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 01/00600

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 26057 A (NOVONORDISK AS) 18 June 1998 (1998-06-18) page 14 -page 21 page 45 -page 49; claims 65-68 -----	9,10,12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/DK 01/00600

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9903962 A	28-01-1999	WO 9903962 A1	28-01-1999
		AU 3884597 A	10-02-1999
		BR 9714808 A	11-12-2001
		EP 1005520 A1	07-06-2000
		JP 2001510233 T	31-07-2001
WO 9826057 A	18-06-1998	AU 5187898 A	03-07-1998
		CN 1245532 A	23-02-2000
		WO 9826057 A1	18-06-1998
		EP 0869167 A2	07-10-1998
		JP 2000507458 T	20-06-2000
		US 6143545 A	07-11-2000
		US 6103505 A	15-08-2000
		EP 0884524 A2	16-12-1998